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Comparative Laboratory Evaluation of the Acute and Chronic Toxicology of Diflubenzuron, Hexaflumuron and Teflubenzuron against II Instar Desert Locust, (*Schistocerca gregaria*) (Orthoptera: Acrididae)

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Abstract: The acute and chronic toxicities of three benzoylphenyl ureas, diflubenzuron, hexaflumuron and teflubenzuron were assessed under laboratory conditions against two-day-old second (II) instar Desert Locust, *Schistocerca gregaria* (Forskål) nymphs. Following exposure by ingestion of a single precise dose applied to short pieces of spring barley, nymphs were monitored for two moults until the fourth (IV) instar. Analysis of acute response data gave three significantly different LD_{50} statistics ($P < 0{\cdot}05$), 68·0, 26·6 and 0·71 μg per nymph respectively for diflubenzuron, hexaflumuron and teflubenzuron. The probit regression slopes also differed significantly, indicating distinct tolerance distributions for the three compounds, the narrowest response being to diflubenzuron and the widest range of response being to teflubenzuron. The timing of death was found to vary between the compounds; most nymphs died during the first moult following treatment with either hexaflumuron or teflubenzuron. However, the majority of nymphs that died after exposure to diflubenzuron did so after completing the first moult after treatment, but before the second. The mean development times of nymphs during the II and especially third (III) instars were significantly longer ($P < 0{\cdot}05$) than those of the controls following exposure to diflubenzuron and hexaflumuron. Teflubenzuron had no significant effect ($P < 0{\cdot}05$) on the duration of the II instar. The potential of the three compounds to control *S. gregaria* populations in the field is discussed with particular reference to the timing and nature of acute and chronic responses.

Key words: diflubenzuron, hexaflumaron, teflubenzuron, insect growth regulator, *Schistocerca gregaria*, desert locust.

1 INTRODUCTION

Locusts and grasshoppers are major pests of agriculture throughout much of the world and are controlled using conventional broad-spectrum insecticides.[1,2] Historically, dieldrin, an organochlorine, which was active both by contact and via ingestion was successfully used to control marching nymphal bands by treating strips of natural vegetation at intervals up to 3 km apart, a technique termed 'barrier spraying'.[3,4] The success of the technique relied on the mobility of nymphal bands which crossed treated barriers and consumed lethal doses of the insecticide. It also, however, required the

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compound to be both toxic to the insect and very persistent. This approach permitted locust control over very large areas at low costs, because only 5% of the land area was treated.[5] Dieldrin is no longer manufactured because of the risk of adverse environmental effects. The lack of a suitable replacement for dieldrin has meant that barrier spraying can no longer be practised; most modern insecticides are non-persistent compared with organochlorines. Insecticidal control of locusts now therefore relies on the use of less persistent compounds that must be applied directly to locusts at much greater cost and reduced overall efficiency.[6] The ground area treated is therefore greater than was the case with dieldrin to achieve the same effect, and it is feared that there may still be considerable risks to non-target species.[7,8] In the fragile, semi-arid and desert ecosystems where these pesticides are used, there is therefore a need to find suitable successors to dieldrin, that will combine effective control using existing application technology with minimal environmental side-effects and at an economically realistic cost.

Diflubenzuron, a benzoylphenyl urea (BPU), is a highly persistent insecticide that acts as a stomach poison but has also significant contact activity, interrupting chitin synthesis in *Schistocerca gregaria* (Forskål) nymphs. It has limited impact on non-target invertebrate populations[9] and is not known to affect vertebrates. It may therefore be a candidate for replacement of organochlorines in locust control.[10,11] Diflubenzuron has since shown up to two months' persistence in sahelian grassland[12] which suggests that it is also potentially suitable for barrier spraying.[13,14] Several other BPUs are now available as candidates in locust control and two of the most promising (hexaflumuron and teflubenzuron) were selected in addition to diflubenzuron for detailed laboratory tests which aimed to compare acute toxicities and rank their activity against II instar *S. gregaria* nymphs. The aim of the work was to establish dose response statistics for all three compounds and monitor sublethal effects, especially on the length of the inter-moult period.

2 MATERIALS AND METHODS

2.1 Insect culture

A laboratory culture of *S. gregaria* was maintained under controlled conditions with a 12:12 h light:dark cycle, a 25°–38°C temperature regime and 40–60% RH. Aluminium cages (38 × 38 × 56 cm) were used to maintain populations of nymphs and adults, illuminated by a single 25-W bulb. The locusts were fed to excess with dry wheat bran and insecticide-free spring barley seedlings grown under glasshouse conditions. This provided the necessary combination of feed for the maintenance of water balance[15] and under these conditions the duration of each developmental stage was relatively predictable; I, II and III instars of four to five days, IV instar of five to seven days, V instar of seven to 10 days and sexually mature adults in 14 days from imaginal moult.

2.2 Insecticides

All three BPUs tested were supplied and tested as oil-based formulations, either suspensions or true solutions. Diflubenzuron ('Dimilin'), supplied by Solvay Duphar BV, Weesp, The Netherlands was formulated as a 450 g litre^{-1} oil dispersable concentrate (ODC); 'Isopar' M, supplied by Exxon Chemical Ltd, Fawley, UK, a non-phytotoxic mineral oil, was used to dilute the ODC. Teflubenzuron ('Nomolt'), was supplied by Shell Research Ltd, Sittingbourne, UK as an experimental 10 g litre^{-1} ULV formulation in cyclohexanone (also used for further dilution). Hexaflumuron ('Consult'), was supplied by DowElanco, Europe as a 250 g litre^{-1} ODC formulation and blank formulation was used for dilution.

2.3 Application procedure

Precise doses of insecticide were applied to short pieces (1.5–2 cm) of young spring barley leaf by an Arnold microapplicator fitted with a Burkard 1-ml gas-tight, glass syringe. The leaves were placed vertically in Petri dishes containing damp, autoclaved silver sand. The quantity of formulation applied to each leaf was limited to a maximum of 2 μl because greater quantities saturated the leaf and ran off into the sand. Consequently a range of different formulation concentrations was prepared to give the required dose range; 10, 40, 60 and 100 g AI litre^{-1} for diflubenzuron, 10, 30 and 100 g AI litre^{-1} for hexaflumaron and 0.25, 0.5, 1 and 10 g AI litre^{-1} for teflubenzuron. Randomly selected two-day-old ('Day 2') II instar nymphs were isolated individually with single treated leaves and covered with clear plastic pot propagator covers (9 × 9 × 15 cm). A minimum of 50 nymphs were used in each treatment to ensure that sufficient nymphs consumed the applied dose within the desired time period. Test insects that consumed the treated leaf within the designated time period were returned to the specified cage for that dose regime with a maximum of 25 nymphs per cage. Higher numbers per cage resulted in cannibalism, especially during the moulting period. A minimum of 50 test insects were also used as controls and were either dosed with blank formulation or given untreated leaves under the same conditions. In order to ensure complete uptake of doses at prescribed times, the test nymphs were normally starved for 3–4 h prior to dosing and occasionally overnight (13–14 h).

2.5 Experimental protocols

Acute responses were evaluated by exposing II instar nymphs to a single dose on Day 2 of the inter-moult period. Between eight and 10 doses were applied to provide an adequate range of responses for subsequent statistical analysis.

Treated and control nymphs were normally monitored once daily until the moult from III to IV instar, i.e. two moults after treatment. This was necessary because at lower dose rates mortality may occur after the first moult.[16,17] Treatments were monitored every day with the numbers of individuals in each instar and the numbers of cadavers in each cage being recorded. Dead insects were classified as either 'in moult' (mortality having occurred during the process of moulting from splitting of exuvium to emergence) or 'post-moult' (the insect successfully moulted, but died during the subsequent inter-moult period). A pre-moult category (for insects that died before any signs of moulting) was used to describe naturally occurring mortality but was not used within the analysis because the BPUs only act during or after moulting.

2.6 Analysis of results

2.6.1 Acute effects

Dose–response data for each BPU were analysed separately by probit analysis (SPSS for Windows, 6.0), following correction for control mortality, to generate regression statistics with 95% confidence intervals from which the effective dose range (giving 1–99% mortality) was predicted. Chi-squared tests were used to confirm homogeneity of the data and, where appropriate, a heterogeneity factor was automatically incorporated into the calculation of confidence intervals.

To examine the statistical significance of any differences between the dose–response relationships of the three BPUs, a parallelism test (in SPSS) was carried out between pairs of regression lines. In order to rank the toxicities of the three compounds, it was necessary to calculate ratios of relative median potency between pairs of BPUs.[18] Two compounds were said to be significantly different ($P < 0{\cdot}05$) when the calculated 95% confidence intervals for the relative median potency did not overlap a value of 1; i.e. both confidence intervals were either >1 or <1.

2.6.2 Mortality classes

χ^2 analysis was used to analyse the frequency distribution of mortality between the classes; in moult and post-moult. The analysis was carried out on count data for each dose and also on the total number recorded in each class for all doses of each BPU. With only two variables and therefore one degree of freedom, Yates' correction was applied.[19]

2.6.3 Chronic effects

The timing of each moult, whether successful or fatal, was recorded for each test insect to determine any treatment effects on the duration of each stage. Data for moulting from II to III were analysed separately from the data for the III–IV moult. The mean number of days to moult (with 95% confidence intervals) was calculated for each treatment. The calculated values identified whether exposure to a BPU had a significant effect on the timing of moulting and hence the duration of an instar.

3 RESULTS

3.1 Acute effects

Probit analysis of $\log_{10}$-transformed dose–response data for the insecticides gave three significantly different LD_{50} values following ingestion by II instar *S. gregaria* (Fig. 1 & Table 1). The nymphs showed variable patterns of susceptibility to each BPU as indicated by the significantly different regression slopes ($\chi^2_2 = 87.38$, $P < 0{\cdot}01$). χ^2 tests performed on transformed dose–response data indicated that diflubenzuron and teflubenzuron data sets were significantly heterogeneous (Table 1). Further investigation revealed that this was a result of formulation effects: when the stock formulations used for the majority of treatments for diflubenzuron and teflubenzuron were analysed separately (diflubenzuron: 100 g AI litre^{-1}; teflubenzuron: 1 g AI litre^{-1}), homogeneity was restored (diflubenzuron: $\chi^2_3 = 4{\cdot}88$, $P > 0{\cdot}1$; teflubenzuron: $\chi^2_3 = 5{\cdot}43$, $P > 0{\cdot}1$) and the calculated LD_{50} 95% confidence intervals (diflubenzuron: 62·02–72·68; teflubenzuron: 0·51–0·83) fell inside those previously estimated.

The relative median potency ratios also differed significantly in all three cases (Fig. 2). It was concluded

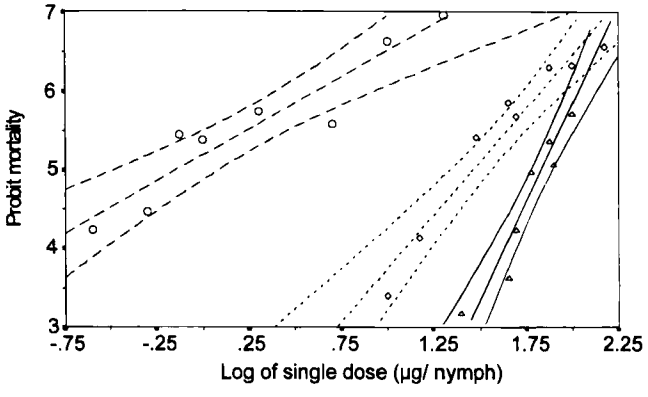

Fig. 1. Dose–response curves for diflubenzuron, hexaflumuron & teflubenzuron to II instars after ingesting a single dose on Day 2. With regression lines and 95% confidence intervals: (—— ^), diflubenzuron; (····· ◇), hexaflumuron; (– – – ○), teflubenzuron. Refer to text for details.

TABLE 1
Probit Statistics for the Acute Dose–Responses of II Instar *Schistocerca gregaria* to Single Ingested Doses of a BPU. Mortality Recorded at or Following Moult to III Instar

BPU	*Probit slope*	*Intercept*	*SE of slope*	LD_{50} (±95% *CI*) (*μg per nymph*)	χ^2 (*d.f.*) *Significance*[a]
Diflubenzuron	5·221	−4·57	0·489	67·97 (60·55–76·31)	14·30 (6)*
Hexaflumuron	2·630	1·25	0·249	26·61 (22·34–30·55)	10·11 (7)[ns]
Teflubenzuron	1·280	5·189	0·154	0·71 (0·29–1·26)	15·38 (6)*

[a] Significance level: ns = not significant; * = $P < 0·05$.

that teflubenzuron was significantly more toxic than either hexaflumuron (37×) or diflubenzuron (100×) and that hexaflumuron was more toxic than diflubenzuron (2×) at the median toxicity levels following a single dose on Day 2 of the II instar.

3.2 Mortality classes

Analysis of the mortality class records at individual dose rates with each BPU indicated significant differences between the insecticides (Table 2). Dose rates with less than a total of nine recorded deaths were not included in the analysis. Mortality following treatment with 50, 75, 80 or 100 μg diflubenzuron ($P < 0·001$), or 60 μg diflubenzuron ($P < 0·05$) occurred predominantly post-moult with only the 150-μg dose (LD_{97}) resulting in a significantly greater number dying during moulting ($P < 0·001$). Following exposure to hexaflumuron or teflubenzuron, nymphs tended to die during the moult.

Overall, significantly more nymphs died post-moult than in moult when treated with a single dose of diflubenzuron. Conversely, significantly more nymphs died in moult than post-moult when treated with a single dose of either hexaflumuron or teflubenzuron (Table 2). The distribution of mortality between the two classes was also found to vary significantly among all three BPUs; diflubenzuron *vs* hexaflumuron ($\chi^2_1 = 121·8$, $P < 0·001$); diflubenzuron *vs* teflubenzuron ($\chi^2_1 = 201·8$, $P < 0·001$); teflubenzuron *vs* hexaflumuron ($\chi^2_1 = 41·5$, $P < 0·001$).

3.3 Inter-moult duration

The duration of the II instar was not significantly different from that of the controls following treatment with 25, 50, 60 or 75 μg of diflubenzuron, 25, 45, 50 or 200 μg of hexaflumuron or following exposure to any dose of teflubenzuron ($P < 0·05$) (Figs 3, 4 & 5). However nymphs dosed with 45 μg of diflubenzuron or 10, 15 or 30 μg of hexaflumuron had significantly shorter II instar periods than controls, whereas those treated with 50, 80, 100 or 150 μg of diflubenzuron, or 75, 100 or 150 μg of hexaflumuron remained as II instars for significantly longer periods. The mean numbers of days taken by diflubenzuron, hexaflumuron and teflubenzuron controls to moult to III instar were not significantly different from each other (5·6 (±0·1); 5·6 (±0·2); 5·8 (±0·2) days respectively).

All treated nymphs that survived to III instar also displayed, with the exceptions of 45 μg diflubenzuron and 15 μg hexaflumuron, a significantly prolonged inter-moult period before moulting to IV instar (Figs 6 & 7). III instar nymphs treated as II instars with 25, 50, 60 or 100 μg diflubenzuron, or 30, 45 or 50 μg hexa-

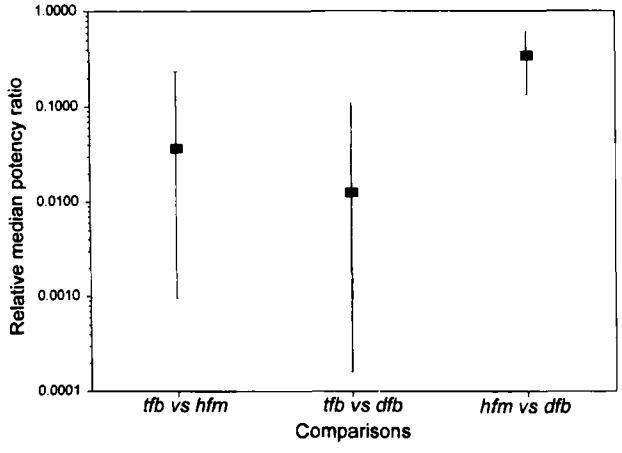

Fig. 2. Relative median potencies with 95% confidence intervals. Values spanning 1 indicate no significant difference in toxicity between compounds (see text).

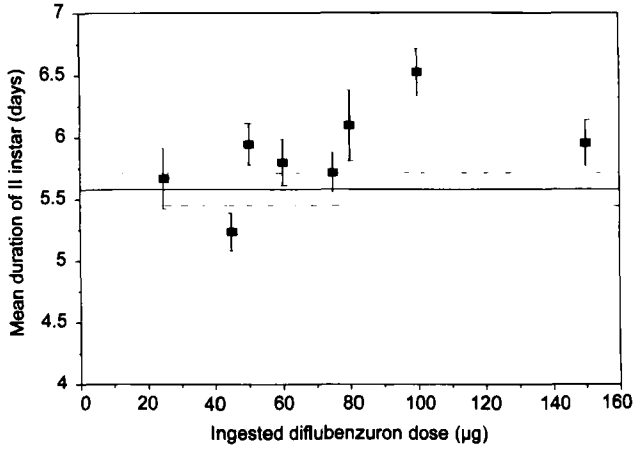

Fig. 3. Mean days (±95% CI) to moult to III instar after a diflubenzuron dose during II instar. Control means and 95% CI shown by full and dashed lines spanning figure.

TABLE 2
χ^2 Analysis of Mortality Classes by Dose Rate

BPU	Dose (μg)	Observed frequencies of mortality classes: In moult	Post-moult	χ^2_1 value Significance[a]
Diflubenzuron	50	1	15	84·5***
	60	19	24	8**
	75	16	32	122·5***
	80	1	22	200***
	100	17	44	338***
	150	28	15	72***
	Totals:	82	152	20***
Hexaflumuron	5	8	1	18***
	30	21	10	50***
	45	29	8	200***
	50	70	37	512***
	75	40	17	242***
	100	31	9	220·5***
	150	62	2	1740·5***
	200	38	2	612·5***
	Totals:	299	86	138***
Teflubenzuron	0·25	9	0	32***
	0·5	12	0	60·5***
	0·75	27	0	338***
	1·0	26	0	312·5***
	2·0	31	0	450***
	5·0	31	0	450***
	10	43	1	840·5***
	20	44	4	760·5***
	Totals:	223	5	207***

[a] Significance of difference between 'in-moult' and 'post-moult': *** $P < 0{\cdot}001$, ** $P < 0{\cdot}01$.

flumuron took up to five days longer to moult to IV instar than untreated controls. Diflubenzuron and hexaflumuron controls took similar times to moult to IV instar (9·1 (±0·1); 9·3 (±0·2) days respectively). The moulting of nymphs from III to IV instars following exposure to teflubenzuron and several diflubenzuron and hexaflumuron doses is not reported because there were either too few/ no survivors from the earlier II–III

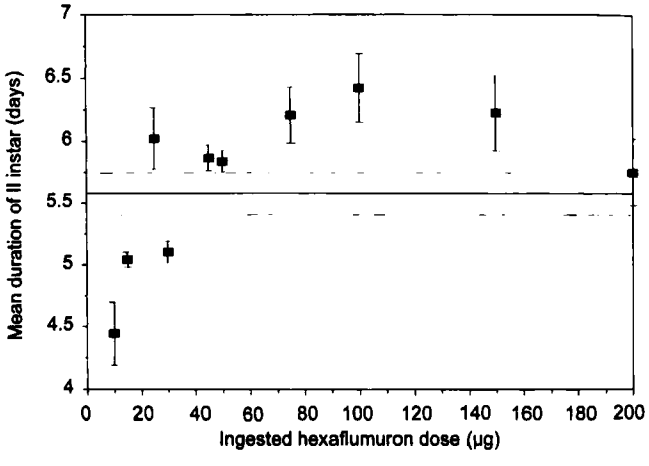

Fig. 4. Mean days (±95% CI) to moult to III instar after a hexaflumuron dose during II instar. Control means and 95% CI shown by full and dashed lines spanning figure.

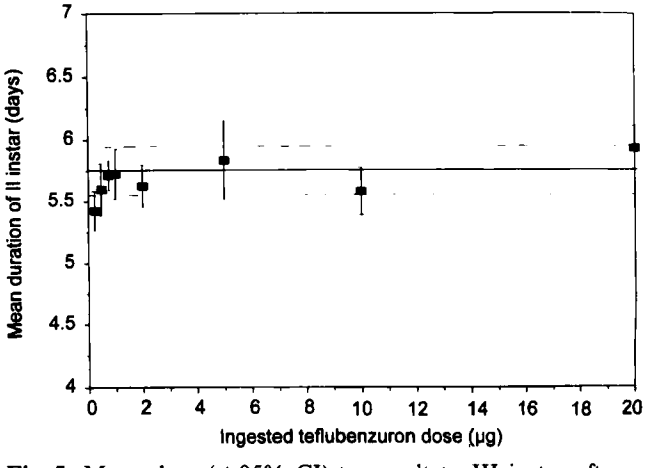

Fig. 5. Mean days (±95% CI) to moult to III instar after a teflubenzuron dose during II instar. Control means and 95% CI shown by full and dashed lines spanning figure.

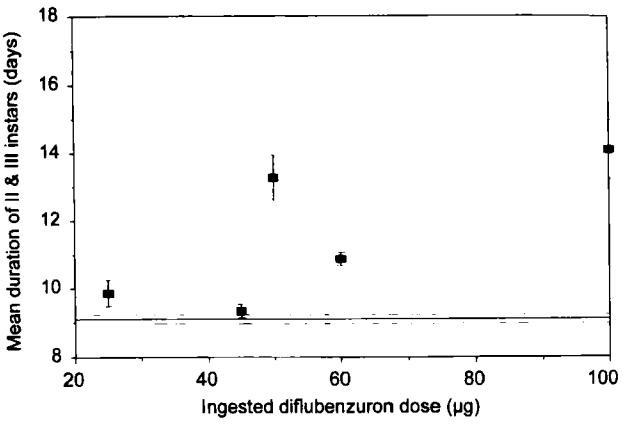

Fig. 6. Mean days (±95% CI) to moult to IV instar after a diflubenzuron dose during II instar. Control means and 95% CI shown by full and dashed lines spanning figure.

moult or the monitoring period did not extend beyond the first moult.

4 DISCUSSION

The varying positions and slopes of the three BPU dose–response curves indicate that these compounds have differing relative potencies and elicit altered tolerance distributions within populations of II instar *S. gregaria* nymphs. Teflubenzuron, which was the most potent compound, had the shallowest slope, indicating that there was a wide tolerance range within the treated population. The breadth in the tolerance distribution compared with the other BPUs indicates greater variability in response within the test population and requires further investigation.

Further differences in acute toxicity between the products were indicated by the contrasting distributions of the mortality classes at similar potency levels. Mortality occurred predominantly 'post-moult' following exposure to diflubenzuron, even at high dose rates. Hexaflumuron caused increased 'in moult' mortality with some occurring 'post-moult', whereas teflubenzuron treatment caused mortality to occur 'in moult'. This suggests that teflubenzuron was the most toxicologically active compound against II instar *S. gregaria*, followed by hexaflumuron and finally diflubenzuron, which showed the most delayed activity. Comparative penetration and elimination studies for the three compounds may reveal why these differences exist.

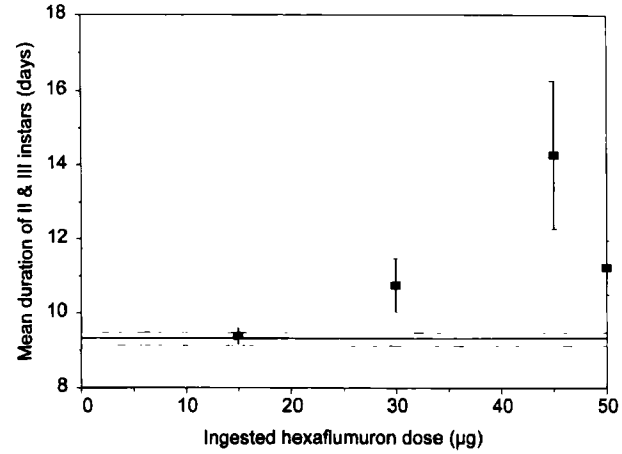

Fig. 7. Mean days (±95% CI) to moult to IV instar after a hexaflumuron dose during II instar. Control means and 95% CI shown by full and dashed lines spanning figure.

The chronic responses that were detected could be of great importance in the field, and significantly affect the final outcome of exposure for locust populations.[17] The increased duration of the II and III instars after exposure to diflubenzuron and hexaflumuron will increase the period of susceptibility and lead to more occasions on which a lethal dose may be consumed by a now weakened nymph. By prolonging development, nymphs may not be able to fledge and mature before the temporary habitat where outbreaks occur has become unsuitable. They will also be exposed to increased predation and parasitism. The resultant mortality would not be directly attributable to BPU effects but the net effect would be the containment and control of the pest population. Other chronic responses that were observed but not quantified included reduced mobility which, under field conditions, may prevent nymphs leaving a treated area and prolong exposure.[20,21] Nymphs were also seen to feed less after exposure, which may contribute to prolonged development. Although reduced feeding may prevent consumption of more treated vegetation, it may be more biologically significant that the nymphs are in a weakened state and have increased susceptibility to further exposure. For an organism that has evolved to exploit temporary habitats by rapid development, mobility and high intrinsic increase rates, reductions in any of these life-history parameters could have major impacts on the epidemiology of *S. gregaria* outbreaks.

The large variations in the acute and chronic toxicities of the three BPUs against II instar *S. gregaria* may be explained by the differing toxicities, retention and metabolism times. These factors appear to be important in other insect species exposed to BPUs, resulting in similar differences in toxicity to those detected here. Diflubenzuron and teflubenzuron are known to be equally effective chitin synthesis inhibitors at the molecular level against *Spodoptera littoralis* Boisd. larvae. However, diflubenzuron is not only metabolised faster than teflubenzuron, it is also excreted faster and has longer penetration times.[22–24] The wide tolerance distributions of nymphs to teflubenzuron is a source of concern because it indicates that some individuals within the test population possess the ability to consistently survive even high doses. In contrast, hexaflumuron has been shown to be less effective at inhibiting chitin synthesis *in vivo* than diflubenzuron, yet it is markedly more active as an insecticide[25] although less so than teflubenzuron.[26] These differences may again result from differential retention times that are a

product of variable rates of metabolism and excretion.[23,24,27]

5 CONCLUSION

Diflubenzuron, hexaflumuron and teflubenzuron were found to be highly toxic to II instar *S. gregaria* when applied as a single dose. All three compounds exhibited important chronic effects such as extended inter-moult periods (diflubenzuron and hexaflumuron) and, though seen but not quantified in this study, reduced mobility and feeding which matched findings in the field[20,21] and laboratory.[17,23] The toxicity of hexaflumuron and teflubenzuron suggests that their use under field conditions against locusts will lead overall to a faster reduction of the target population than diflubenzuron (assuming equivalent persistence) although the time taken to achieve initial effects will remain similar. Ecotoxicological investigations must be carried out alongside efficacy trials. This study has ranked the toxicities of three BPUs following a single dose, which, although useful in providing a benchmark, does not accurately reflect exposure and uptake in the field. A more realistic approach will be needed to examine the duration and the timing of exposure, both of which are liable to be critical for BPUs that do not have immediate effects and show variable persistence within the target organism.

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